RESEARCH ARTICLE

Internalization of *Pseudomonas aeruginosa* Strain PAO1 into Epithelial Cells Is Promoted by Interaction of a T6SS Effector with the Microtubule Network

Thibault G. Sana,[a]* Christoph Baumann,[b] Andreas Merdes,[c] Chantal Soscia,[a] Thomas Rattei,[d] Abderrahman Hachani,[a,e] Cerith Jones,[e] Keiryn L. Bennett,[b] Alain Filloux,[e] Giulio Superti-Furga,[b] Romé Voulhoux,[a] Sophie Bleves[a]

CNRS & Aix-Marseille Université, Laboratoire d'Ingénierie des Systèmes Macromoléculaires (UMR7255), Institut de Microbiologie de la Méditerranée, Marseille, France[a]; Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria[b]; Centre de Biologie du Développement, Université Paul Sabatier, Toulouse, France[c]; Department of Microbiology and Ecosystem Science, University of Vienna, Vienna, Austria[d]; Department of Life Sciences, MRC Centre for Molecular Bacteriology and Infection, Imperial College London, London, United Kingdom[e]

* Present address: Thibault G. Sana, Department of Microbiology and Immunology, Stanford University Medical Center, Stanford, California, USA.

ABSTRACT **Invasion of nonphagocytic cells through rearrangement of the actin cytoskeleton is a common immune evasion mechanism used by most intracellular bacteria. However, some pathogens modulate host microtubules as well by a still poorly understood mechanism. In this study, we aim at deciphering the mechanisms by which the opportunistic bacterial pathogen *Pseudomonas aeruginosa* invades nonphagocytic cells, although it is considered mainly an extracellular bacterium. Using confocal microscopy and immunofluorescence, we show that the evolved VgrG2b effector of *P. aeruginosa* strain PAO1 is delivered into epithelial cells by a type VI secretion system, called H2-T6SS, involving the VgrG2a component. An *in vivo* interactome of VgrG2b in host cells allows the identification of microtubule components, including the γ-tubulin ring complex (γTuRC), a multiprotein complex catalyzing microtubule nucleation, as the major host target of VgrG2b. This interaction promotes a microtubule-dependent internalization of the bacterium since colchicine and nocodazole, two microtubule-destabilizing drugs, prevent VgrG2b-mediated *P. aeruginosa* entry even if the invasion still requires actin. We further validate our findings by demonstrating that the type VI injection step can be bypassed by ectopic production of VgrG2b inside target cells prior to infection. Moreover, such uncoupling between VgrG2b injection and bacterial internalization also reveals that they constitute two independent steps. With VgrG2b, we provide the first example of a bacterial protein interacting with the γTuRC. Our study offers key insight into the mechanism of self-promoting invasion of *P. aeruginosa* into human cells via a directed and specific effector-host protein interaction.**

IMPORTANCE **Innate immunity and specifically professional phagocytic cells are key determinants in the ability of the host to control *P. aeruginosa* infection. However, among various virulence strategies, including attack, this opportunistic bacterial pathogen is able to avoid host clearance by triggering its own internalization in nonphagocytic cells. We previously showed that a protein secretion/injection machinery, called the H2 type VI secretion system (H2-T6SS), promotes *P. aeruginosa* uptake by epithelial cells. Here we investigate which H2-T6SS effector enables *P. aeruginosa* to enter nonphagocytic cells. We show that VgrG2b is delivered by the H2-T6SS machinery into epithelial cells, where it interacts with microtubules and, more particularly, with the γ-tubulin ring complex (γTuRC) known as the microtubule-nucleating center. This interaction precedes a microtubule- and actin-dependent internalization of *P. aeruginosa*. We thus discovered an unprecedented target for a bacterial virulence factor since VgrG2b constitutes, to our knowledge, the first example of a bacterial protein interacting with the γTuRC.**

Received 26 April 2015 **Accepted** 6 May 2015 **Published** 2 June 2015

Citation Sana TG, Baumann C, Merdes A, Soscia C, Rattei T, Hachani A, Jones C, Bennett KL, Filloux A, Superti-Furga G, Voulhoux R, Bleves S. 2015. Internalization of *Pseudomonas aeruginosa* strain PAO1 into epithelial cells is promoted by interaction of a T6SS effector with the microtubule network. mBio 6(3):e00712-15. doi:10.1128/mBio.00712-15.

Invited Editor Dara W. Frank, Medical College of Wisconsin **Editor** Eric J. Rubin, Harvard School of Public Health

Address correspondence to Sophie Bleves, bleves@imm.cnrs.fr.

One of the key requirements for pathogenic bacteria to establish an infection and persist in an immunocompetent host is successful avoidance of innate defense mechanisms. A frequently used strategy for immune evasion employed by invasive bacteria is to direct their uptake into host cells that are not normally phagocytic, including epithelial and endothelial cells lining mucosal surfaces and blood vessels, respectively. Presumably, this invasion tactic ensures a protected cellular niche for the pathogen to replicate or persist, and in some instances, it can also allow transit into deeper tissues through tight epithelial cell barriers. The most common way to induce phagocytosis involves the subversion of the actin remodeling machinery to manipulate the cytoskeleton into producing actin-rich cell surface projections specifically promoting the engulfment of the adherent bacteria (1, 2). Although most

invasive bacteria utilize host actin for entry, some also appear to modulate the dynamics of host microtubules (3). Examples include enteropathogenic and enteroinvasive *Escherichia coli* (4), *Haemophilus influenzae* (5), *Campylobacter jejuni*, and *Citrobacter freundii* (6), *Neisseria gonorrhoeae* (7), or *Burkholderia cepacia* complex (BCC) (8). Although microtubules are not involved in the internalization processes, such as phagocytosis, treatment of epithelial cells with microtubule-destabilizing agents like colchicine or nocodazole decreases the number of internalized bacteria of these species. However, the molecular basis of microtubule-dependent invasion is still poorly understood.

Pseudomonas aeruginosa is an opportunistic bacterium of humans leading to various infections in immunocompromised people, including bacteremia, sepsis, pneumonia, and wound and skin infections (9). This pathogen is also responsible for chronic lung infections and lethal consequences in patients with cystic fibrosis. While considered an extracellular pathogen, *P. aeruginosa* is able to enter nonphagocytic cells such as epithelial and endothelial cells (10, 11). Different isolates vary in their internalization efficiency into cultured mammalian cells (12). Among them, the invasive strains, which are the most common in *P. aeruginosa* species, possess the ExoS effector, while the cytotoxic and hence noninvasive ones lack *exoS* and instead encode the acute cytotoxin ExoU, which can quickly kill cells (13–15). However, this ability of internalization has been maintained over the course of *P. aeruginosa* evolution, indicating a fundamental role in the infection, notably in cornea invasion (16). *P. aeruginosa* preferentially infects damaged epithelial tissues and exploits the epithelial cell polarization machinery (11, 13, 17, 18). In this process, the binding of the bacterium to the cell surface activates a central host-signaling molecule, phosphatidylinositol 3-kinase (PI3K), required for synthesis of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and for activation of a downstream effector, the Ser/Thr kinase Akt (19). The result is that the bacterium transforms apical into basolateral membrane, creating a local microenvironment that facilitates its colonization and entry into the mucosal barrier.

We have demonstrated that among the various factors facilitating internalization of *P. aeruginosa*, the H2 type VI secretion system (H2-T6SS) is involved in the uptake of the bacterium by eukaryotic cells (20). An H2-T6SS mutant indeed displays a significant decrease in HeLa cell invasion and, concomitantly, a reduced Akt phosphorylation in infected lung epithelial cells. Recently, Jiang and colleagues showed that the H3-T6SS also mediates *P. aeruginosa* PAO1 uptake and that H2-T6SS and H3-T6SS can compensate for each other under certain growth conditions (21). Two phospholipases D, PldA (Tle5) and PldB, which depend on the H2-T6SS and H3-T6SS, respectively, are involved in Akt binding. The type VI secretion machinery allows Gram-negative bacteria to interact with either eukaryotes or bacteria and deliver effector proteins into target cells upon contact (22–25). The T6SS puncturing device is structurally related to the bacteriophage contractile tail and includes the hemolysin-coregulated protein (Hcp) and valine glycine repeat G (VgrG) proteins. A trimer of VgrG proteins may fulfill the role of the phage T4 $(gp27/gp5)_3$ complex at the tip of the tail (26). Some of the T6SS machineries, like those of *Vibrio cholerae* (27–29) and *P. aeruginosa* (20, 21, 30), permit interaction with both eukaryotic and bacterial target cells. PldA and PldB are even transkingdom effectors since they target both prokaryotic and eukaryotic cells; each of them triggers killing of bacterial competitors and internalization of *P. aeruginosa* into nonphagocytic cells (21, 30, 31). The first discovered antieukaryotic T6SS effectors belong to a subclass of VgrG proteins, called evolved VgrGs, containing C-terminal extensions with specialized intracellular functions. The C-terminal extension, an actin cross-linking domain in VgrG1 of *V. cholerae* (27, 32) or an ADP-ribosyl transferase domain in VgrG1 of *Aeromonas hydrophila* (33), promotes a cytotoxic effect toward eukaryotic host cells by disrupting the actin cytoskeleton. These evolved VgrGs have a dual function, playing a structural role in the puncturing device as well as an effector function (34). The second type of antieukaryotic T6SS effector is an independent toxin targeted to the host cell. The N-terminal pleckstrin homology domain of VasX from *V. cholerae* may be implicated in binding the host membrane lipids and thus in the interference with host phosphoinositide metabolism and signaling (35). Likewise PldA and PldB bind Akt to promote *P. aeruginosa* internalization (21).

Here we investigated which H2-T6SS-related VgrG protein enables *P. aeruginosa* strain PAO1 to enter epithelial cells. We showed that VgrG2b is delivered by the H2-T6SS machinery into epithelial cells, where it interacts with microtubule components, including the γ-tubulin ring complex (γTuRC), which precedes a microtubule- and actin-dependent internalization of *P. aeruginosa*. We thus discovered a novel target for a bacterial virulence factor since VgrG2b constitutes, to our knowledge, the first example of a bacterial protein interacting with the γTuRC.

RESULTS

VgrG2a and VgrG2b are important for *P. aeruginosa* internalization. While the majority of T6SS loci contain in their immediate proximity *hcp* and *vgrG* genes (36), these are absent at the vicinity of the *P. aeruginosa* strain PAO1 H2-T6SS gene cluster (Fig. 1A). Based on phylogenetic clustering, two sets of orphan Hcp and VgrG proteins have been proposed as likely candidates to represent the two proteins required for the H2-T6SS function (36–38). These are proteins encoded by the *hcp2a-vgrG2a* (PA1512-PA1511) and *hcp2b-vgrG2b* (PA0263-PA0262) loci and may be part of larger operons (Fig. 1A). They may be constituents of the so-called "*vgrG* islands" (36), very likely acquired by horizontal gene transfer and frequently linked to genes encoding type VI lipase effector (Tle) with putative a GxSxG catalytic motif and potential lipase activity (30). In the *vgrG2a* island, the linked product of PA1510 has a putative PGAP1-like domain (PF07819) and belongs to the Tle4 family (30). In the *vgrG2b* island, the PA0260-encoded protein has an α-β hydrolase domain (PF00561) and has been classified in the Tle3 family (30). The products of PA1509 and PA0259 have been proposed to be the putative immunity proteins of Tle4 and Tle3, respectively (30). The two Hcp2 proteins are 100% identical, while the VgrG2a and VgrG2b proteins are not identical in size but share 93% identity over their first 828 residues (Fig. 1B). The N-terminal 581 residues of these two proteins contain the VgrG domain, homologous to $(gp27)_3$-$(gp5)_3$ phage-tail proteins, which is followed by a domain of unknown function (DUF2345) present in many VgrG proteins. VgrG2b contains an extended C-terminal extension with a putative zinc-dependent metallopeptidase domain (PS00142) and has been thus considered an evolved VgrG (32).

To demonstrate a functional connection between H2-T6SS and these Hcp2-VgrG2 orphans, we took advantage of the role of the H2-T6SS machinery in *P. aeruginosa* internalization into nonphagocytic cells (20). We paid special attention to cultivating

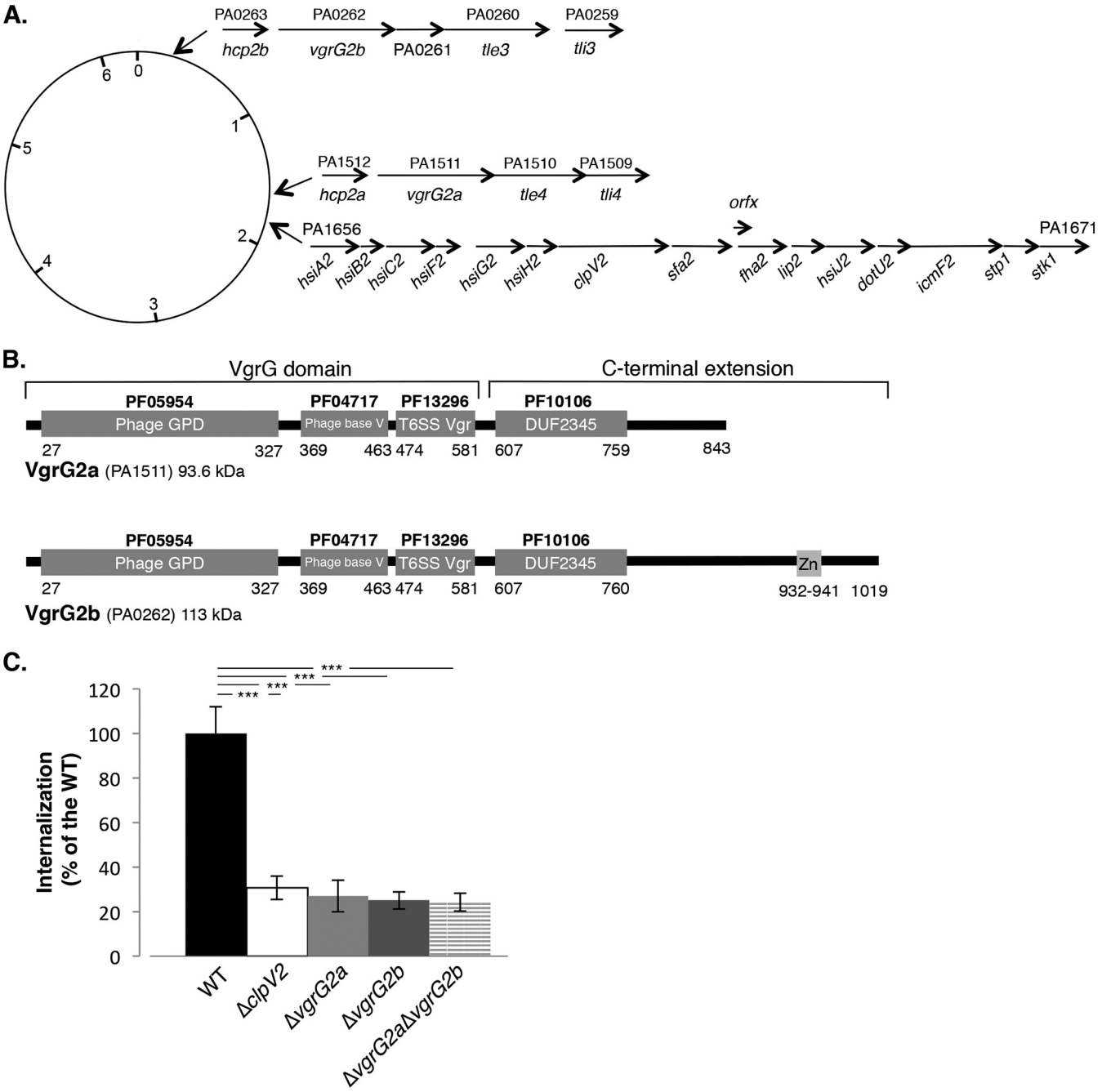


FIG 1 VgrG2a and VgrG2b are likely components of the H2-T6SS machinery. (A) *vgrG2a* and *vgrG2b* island organization. The numbers on the map indicate base pair numbers in millions. The genes are labeled with the given name (i.e., *vgrG2a*) or are indicated by their annotation number (e.g., PA0261). (B) VgrG2a and VgrG2b domain architecture. The first 581 residues carry the VgrG domain, consisting of the phage GPD (phage late control gene D protein, PF05954), phage base V (phage-related baseplate assembly protein, PF04717), and T6SS Vgr (putative type VI secretion system Rhs element, PF13296) domains, homologous to gp27 and gp5 phage tail proteins. This is followed by a conserved domain of uncharacterized proteins (DUF2345, PF10106) present in many VgrG proteins. VgrG2b possesses an additional C-terminal extension of 186 residues, with a putative zinc-dependent metallopeptidase pattern (LFIHEMTHVW signature, PS00142). (C) Bacterial invasion (gentamicin protection assays) of HeLa cells upon 3 h of infection at an MOI of 10 with *P. aeruginosa* strains grown at the transition between the exponential and stationary phases in Trypticase soy broth (TSB) at 37°C. The percentage of invasion of PAO1Δ*clpV2*, PAO1Δ*vgrG2a*, PAO1Δ*vgrG2b*, or PAO1Δ*vgrG2a* Δ*vgrG2b* was normalized to the WT PAO1 strain, which represents an average of 1.3×10^5 CFU of internalized bacteria per well (2.5×10^5 HeLa cells). Each experiment was performed a minimum of three times in triplicate. The error bars indicate standard deviations. ***, $P \leq 0.001$.

bacteria in the exponential phase before infecting HeLa cells (20) since H3-T6SS can take over H2-T6SS function in internalization when cells are grown in the stationary phase (21). This growth-phase-dependent compensation is explained by the expression patterns of the two gene clusters. Quorum sensing indeed induces the two gene clusters in the exponential phase, but whereas H2-T6SS expression is maximal during the log- to stationary-phase transition, H3-T6SS is efficiently expressed later in the stationary

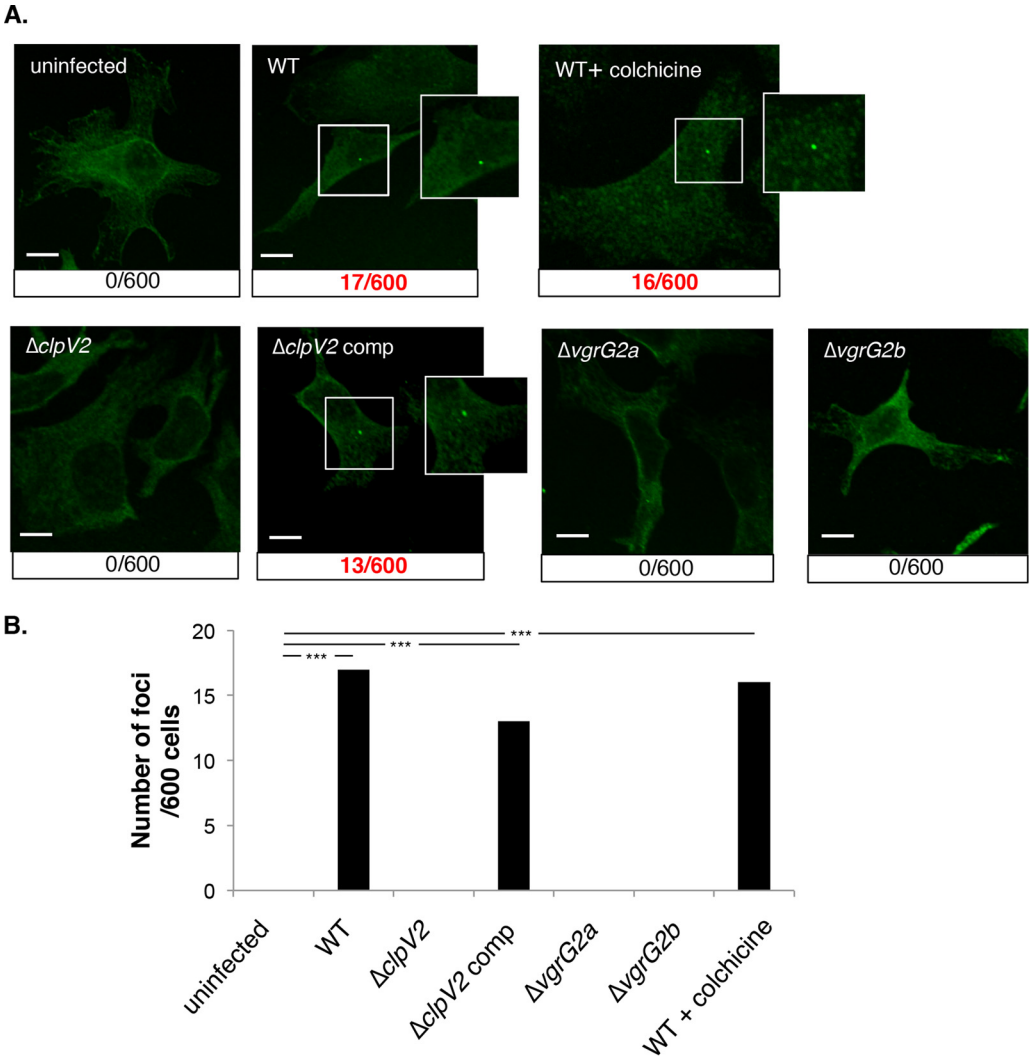


FIG 2 VgrG2b is delivered in the host cell upon *P. aeruginosa* infection. Shown are results from confocal laser-scanning microscopy of HeLa cells infected or not (uninfected) with *P. aeruginosa* PAO1 (WT) and the mutant strains indicated (A). The *clpV2* mutation was complemented by expression in *trans* of *clpV2* from the pHM49 plasmid (Δ*clpV2* comp). VgrG2b was labeled with VgrG2b antiserum and detected with secondary anti-IgG antibodies conjugated to Cy3. Representative images of individual cells after counting of 600 cells from four independent experiments are presented. The black and red numbers indicate the number of cells in which VgrG2b is detected out of the total cell numbers observed. When indicated, 10 μg/ml of colchicine was added 30 min before HeLa cell infection. Scale bar, 5 μm. A graphical representation of the number of VgrG2b foci observed after counting of 600 cells is shown in panel B. ***, $P \leq 0.001$ by χ^2 test.

phase (39). As expected, upon *P. aeruginosa* infection of HeLa cells, only a few of the PAO1Δ*clpV2* bacteria, an H2-T6SS-defective strain, were internalized in comparison to the wild-type (WT) PAO1 strain (Fig. 1C) (20, 21). The same impairment (about 75% decrease) was observed for the PAO1Δ*vgrG2a*, PAO1Δ*vgrG2b*, and PAO1Δ*vgrG2a*Δ*vgrG2b* mutant strains, whereas the adherence of the mutant strains was not affected (see Fig. S1A in the supplemental material). The requirement of VgrG2a, VgrG2b, and the H2-T6SS machinery for the internalization was confirmed in a more physiologically relevant cell line, A549 human alveolar epithelial cells (see Fig. S1B). This demonstrates that internalization of *P. aeruginosa* requires a functional H2-T6SS and the two *vgrG2* products (Fig. 1A and B). These results show a functional link between VgrG2a, VgrG2b, and the H2-T6SS machinery and identify VgrG2a as a component and the evolved VgrG2b as a very likely cognate effector.

VgrG2b is delivered into host cells by the H2-T6SS machinery. We used confocal laser scanning microscopy to investigate whether VgrG2b is delivered into epithelial cells upon *P. aeruginosa* infection. In order to differentiate between the two VgrG2s when using immune detection, an antiserum specific to the VgrG2b C-terminal extension was developed (see Fig. S2A and B in the supplemental material). The examination of multiple individual cells revealed the presence of VgrG2b within some HeLa cells infected with the WT PAO1 strain (VgrG2b detection in 17 cells out of ≅600 observed cells) that are absent in the uninfected cells (Fig. 2A and B). When observed, the VgrG2b signals were not evenly distributed throughout the infected cells but instead were present as foci within the cytoplasm. On average, we observed one VgrG2b fluorescent dot per infected cell and in 3 cells more than one spot. Image acquisition at various focal levels of the epithelial cells confirmed that VgrG2b signal was within the

infected cells (see Fig. S2C). Importantly, the VgrG2b intracellular localization was dependent on a functional H2-T6SS machine since foci were not detected upon infection with a *clpV2* mutant (in no cell out of ≅600 observed cells) (Fig. 2), while VgrG2b was readily detected in the mutant bacteria (see Fig. S2D, left panel). Complementation in *trans* of the *clpV2* mutation restored intracellular detection (in 13 cells out of ≅600 observed cells) (Fig. 2A and B). The very low level of intracellular VgrG2b (≈2.5%) might reflect the low level of internalized *P. aeruginosa* that we and others have previously observed (≈1.7% [12, 20]).

Moreover, the infection with a *vgrG2a* mutant that produces VgrG2b (see Fig. S2A and D, left panel, in the supplemental material) did not allow any intracellular immunodetection of VgrG2b, suggesting that VgrG2a may be required for VgrG2b delivery into host cells (Fig. 2A and B), which is in line with its involvement as an H2-T2SS component. As we have not ruled out that VgrG2a can also be injected, a chromosomally encoded $VgrG2a_{V5}$ translational fusion was engineered in order to specifically detect VgrG2a. Whereas $VgrG2a_{V5}$ was synthesized by *P. aeruginosa* (see Fig. S2D, right panel), we have been unable to detect it in infected cells (see Fig. S2E). This suggests either that VgrG2a is not targeted to the eukaryotic host cell, or the technique we used is not sensitive enough to detect VgrG2a translocation. The VgrG2a-dependent delivery of VgrG2b is in agreement with the heteromultimeric complexes formed by VgrGs (32, 38), although we have not been able to observe multimeric complexes containing VgrG2a and VgrG2b. Nevertheless this observation constitutes another piece of evidence that the orphan *vgrG2a* and *vgrG2b* islands are linked to the H2-T6SS cluster (Fig. 1A).

Altogether these data show that upon contact with the target cells, VgrG2b is delivered into the host cytoplasm by the H2-T6SS machinery involving VgrG2a. This is fully in agreement with the phylogenetic placement of VgrG2b as an evolved VgrG, an effector with putative activity in the infected mammalian cells.

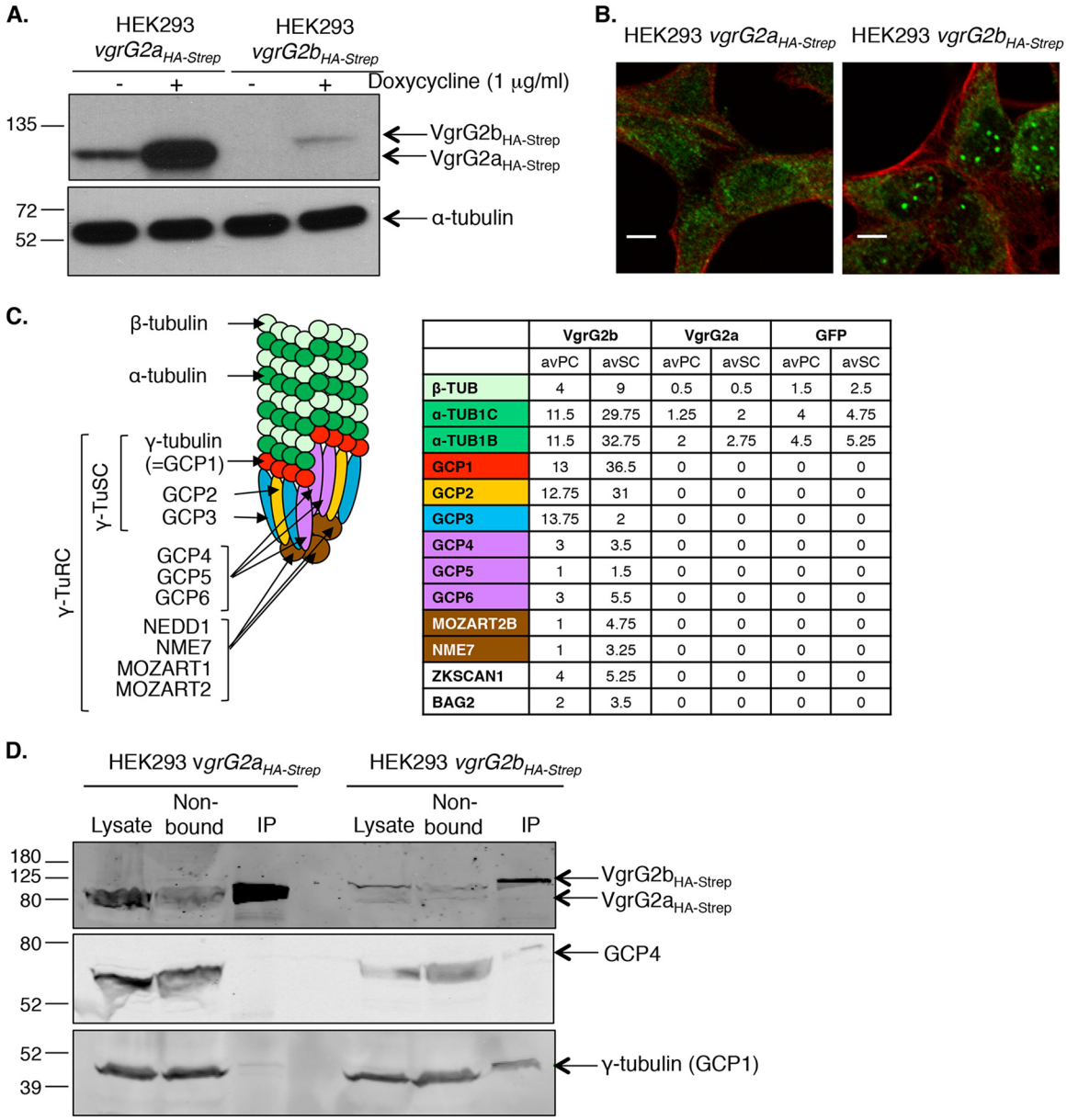
An *in vivo* interactome approach identifies γTuRCs as partners of VgrG2b. In order to identify the host intracellular partners for VgrG2b, we made use of a tandem affinity purification-mass spectrometry (TAP-MS) procedure (40, 41). First a HEK293 cell line producing $VgrG2b_{HA\text{-}Strep}$ upon doxycycline induction was engineered (Fig. 3A and B). Moreover, to be able to exclude proteins copurifiying in a nonspecific manner, a cell line producing hemagglutinin-streptavidin (HA-Strep)-tagged green fluorescent protein (GFP) was used. Two-step tandem affinity purification for stringent enrichment of interacting proteins was performed (41). The analysis indicated that the TAP-tagged VgrG2b bound the α-tubulins (α-TUB1C and α-TUB1B) and β-tubulin, the microtubule subunits, which were also unspecifically recovered to a lesser extent with the control GFP (Fig. 3C; see Fig. S3 in the supplemental material). Interestingly the TAP-tagged VgrG2b also copurified specifically the full complement of proteins constituting the γ-tubulin complexes, such as γ-tubulin (GCP1), GCP2, and GCP3 of the γ-tubulin small complex(γTuSC), as well as GCP4, -5, and -6, NME7, and Mozart2B of the γ-tubulin ring complex (γTuRC) (Fig. 3C; see Fig. S3). The γ-tubulin complexes regulate microtubule nucleation, providing spatial and temporal control over the initiation of microtubule growth (42). In several animal cells, these nucleation sites are the centrosomes, which are organized around a pair of centrioles and serve as the central anchoring point for microtubules within the cell.

As a specific negative control, the TAP-MS procedure was also applied to HEK293 cells producing the closely related $VgrG2a_{HA\text{-}Strep}$ (Fig. 3A and B). This did not lead to the copurification of any specific proteins (Fig. 3C and Fig. S3), whereas $VgrG2a_{HA\text{-}Strep}$ was much more produced than $VgrG2b_{HA\text{-}Strep}$ (Fig. 3A), suggesting that VgrG2b rather than VgrG2a has specificity for eukaryotic host interactions. Furthermore, this confirms the specificity of the detected VgrG2b interactions and suggests that the interaction with γ-tubulin complex proteins is likely mediated by the C-terminal extension of VgrG2b, which is absent in VgrG2a (Fig. 1B). It is also noteworthy that the spatial distributions of the two TAP-tagged VgrG2s produced in the HEK293 cell line are very different. Whereas $VgrG2a_{HA\text{-}Strep}$ had a diffuse localization throughout the cytoplasm (Fig. 3B), $VgrG2b_{HA\text{-}Strep}$ was observed as several foci, similar to the pattern seen following infection of cells with *P. aeruginosa* (Fig. 2A). This focal accumulation may reflect the targeting of VgrG2b to an organelle or a cellular complex.

To confirm the specific VgrG2b interaction with the γ-tubulin complex and exclude an indirect interaction through microtubules, we performed coimmunoprecipitation from HEK293 cell lysates under microtubule-depolymerizing conditions (43, 44) (Fig. 3D). The anti-HA antibodies specifically coprecipitated GCP4 and γ-tubulin (GCP1) from γTuRC, together with $VgrG2b_{HA\text{-}Strep}$. Although $VgrG2a_{HA\text{-}Strep}$ was efficiently precipitated by the anti-HA antibodies, none of the tested proteins of the γ-tubulin complex coimmunoprecipitated. This suggests a role of the C-terminal extension of VgrG2b absent in VgrG2a in the interaction with the γTuRC, and this is independent of the microtubules themselves since microtubules are not polymerized under the conditions used in this experiment.

The T6SS-dependent internalization of *P. aeruginosa* is mediated by both microtubule and actin cytoskeleton. Since VgrG2b interacts with microtubule components, we next examined the requirement of microtubules for the H2-T6SS-dependent uptake of *P. aeruginosa* by epithelial cells. Although many pathogenic bacteria modulate the actin cytoskeleton of eukaryotic cells to facilitate the invasion of epithelial cells, some pathogens are capable of modulating the dynamics of host microtubules as well. Few studies have examined the requirement for microtubules during *P. aeruginosa* PAO1 strain internalization, and it seems uncertain since it is independent for corneal epithelial cell infection (18) and dependent for middle ear epithelial cells (45). Hence we studied *P. aeruginosa* uptake by HeLa cells in the presence of colchicine or nocodazole, two microtubule-destabilizing agents. Colchicine inhibited *P. aeruginosa* uptake in a dose-dependent manner (Fig. 4A). Importantly, the drug affected neither viability nor growth of the bacteria (data not shown) and moreover did not influence bacterial adherence (see Fig. S4A in the supplemental material). A similar decrease in cell invasion was observed following pretreatment of HeLa cells with various concentrations of nocodazole (see Fig. S4B). However, as expected from the literature (12, 18, 46), the internalization still requires the actin cytoskeleton since treatment with cytochalasin D (20 μM), which causes microfilament depolymerization, decreased *P. aeruginosa* uptake by HeLa cells (see Fig. S4C).

To determine if *P. aeruginosa* delivers VgrG2b in host cells from the outside or once the bacterium has been internalized, we studied the VgrG2b foci in HeLa cells pretreated with colchicine at 10 μg/ml, a concentration inhibiting internalization (Fig. 4A). Since VgrG2b was still observed inside infected HeLa cells in the



	VgrG2b		VgrG2a		GFP	
	avPC	avSC	avPC	avSC	avPC	avSC
β-TUB	4	9	0.5	0.5	1.5	2.5
α-TUB1C	11.5	29.75	1.25	2	4	4.75
α-TUB1B	11.5	32.75	2	2.75	4.5	5.25
GCP1	13	36.5	0	0	0	0
GCP2	12.75	31	0	0	0	0
GCP3	13.75	2	0	0	0	0
GCP4	3	3.5	0	0	0	0
GCP5	1	1.5	0	0	0	0
GCP6	3	5.5	0	0	0	0
MOZART2B	1	4.75	0	0	0	0
NME7	1	3.25	0	0	0	0
ZKSCAN1	4	5.25	0	0	0	0
BAG2	2	3.5	0	0	0	0

FIG 3 VgrG2b interacts with γ-tubulin complexes in host cells. Immunodetection of ectopically produced VgrG2a$_{HA-Strep}$ and VgrG2b$_{HA-Strep}$ in HEK293 cells by Western blotting (A) or confocal microscopy (B). Doxycycline (1 μg/ml) was added during 24 h (A) or 16 h (B) for the induction of *vgrG2a$_{HAStrep}$* and *vgrG2b$_{HAStrep}$*. In panel A, VgrG2 proteins are detected with anti-VgrG2a antiserum, and α-tubulin immunodetection is used as a loading control. Molecular masses in kilodaltons are indicated on the left. In panel B, VgrG2 proteins are immunostained in green with anti-HA antiserum and α-tubulin in red. Scale bar, 5 μm. Proteins identified specifically in VgrG2b pulldowns are shown in panel C. Shown are average peptide counts (avPC) and average spectral counts (avSC) reflecting enrichment of proteins from two independent experiments, each processed in two technical replicates measured by LC-MS/MS. A full table including all detailed MS measurements is provided in Fig. S3 in the supplemental material. The most widely accepted model for the mechanism of γTuRC-based nucleation is presented and based on that of Kollman et al. (42). (D) Anti-HA coimmunoprecipitation assay in HEK293 cells producing VgrG2a$_{HA-Strep}$ or VgrG2b$_{HA-Strep}$. Shown is immunodetection of VgrG2a/2b$_{HA-Strep}$ with anti-HA antiserum, GCP4 with anti-GCP4, and γ-tubulin with anti-GCP1 in cell lysate and nonbound fraction and immunoprecipitate (IP). Molecular masses in kilodaltons are indicated on the left.

presence of colchicine (in 16 cells out of ≅600 observed cells [Fig. 2]) and while *P. aeruginosa* remained extracellular (Fig. 4A), we concluded that VgrG2b injection by the H2-T6SS machinery precedes the internalization of the bacterium. However, by using confocal microscopy, we have been unable to detect both VgrG2b and entering or intracellular bacteria. VgrG2b delivery by extracellular bacteria is thus a separate step from *P. aeruginosa* uptake.

We further hypothesized that if *P. aeruginosa* internalization triggered by the H2-T6SS machinery relies exclusively on a VgrG2b-mediated modulation of the microtubule cytoskeleton, colchicine treatment would not have any additional effect in an H2-T6SS mutant background (Fig. 4B). We indeed observed that the internalization defect of several H2-T6SS mutants (Δ*clpV2*, Δ*vgrG2a*, Δ*vgrG2b*, or Δ*vgrG2a*Δ*vgrG2b*) is not increased by col-

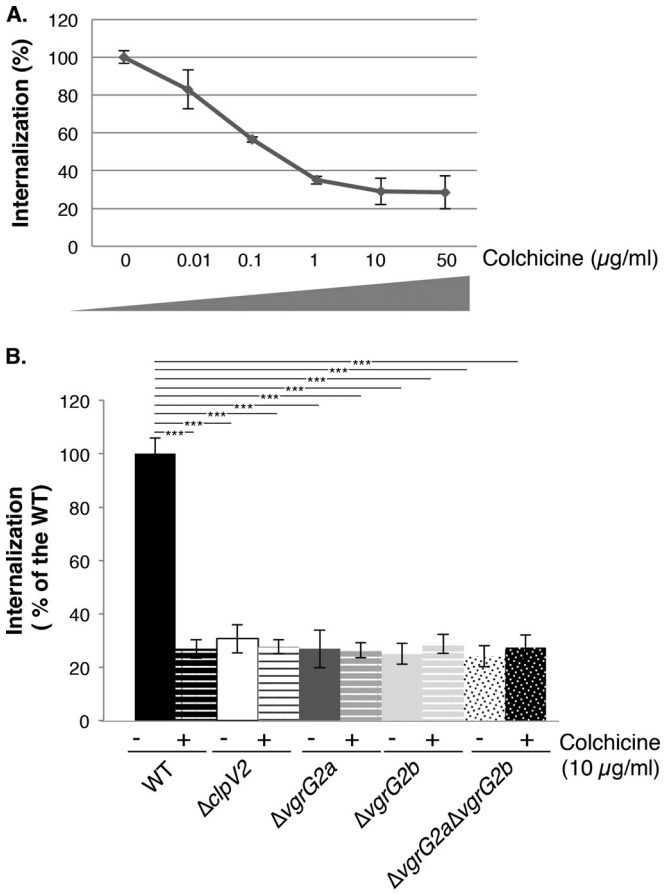


FIG 4 Colchicine prevents H2-T6SS-dependent internalization of *P. aeruginosa*. (A) Internalization of *P. aeruginosa* by HeLa cells with 0, 0.01, 0.1, 1, 10, and 50 μg/ml of colchicine added 30 min before infection with WT *P. aeruginosa* PAO1 for 3 h at an MOI of 10. The percentage of invasion was normalized to PAO1 in the absence of colchicine. (B) Internalization of different *P. aeruginosa* PAO1 mutants (at an MOI of 10) by HeLa cells with (+) or without (−) 10 μg/ml of colchicine added 30 min before infection for 3 h. The percentage of invasion was normalized to PAO1, which represents an average of 1.3×10^5 CFU of internalized bacteria per well (2.5×10^5 HeLa cells). Each experiment was performed a minimum of three times in triplicate. The error bars indicate standard deviations. ***, $P \leq 0.001$.

chicine treatment of HeLa cells. We also observed that use of colchicine at a concentration of 10 μg/ml results in the same magnitude of internalization impairment (around 75%) as that in a strain with a mutation in the H2-T6SS. Based on these data, we propose that the H2-T6SS-dependent translocation of VgrG2b precedes and facilitates *P. aeruginosa* entry by microtubules and still requires actin microfilaments.

Bypassing the injection step by intracellular production of VgrG2b. We next investigated whether intracellular production of VgrG2b in the host prior to infection could boost the internalization of *P. aeruginosa*, even if other factors mediating *P. aeruginosa* uptake can be limiting (i.e., adhesins or PldA). We took advantage of the stable cell line overproducing $VgrG2b_{HA\text{-}Strep}$ and performed infection of these HEK293 cells with *P. aeruginosa* (Fig. 5). The induction of $vgrG2b_{HA\text{-}Strep}$ expression by doxycycline leads to an approximately 3-fold increase in the number of WT bacteria recovered in HEK293 cells after infection (compare bars 1 and 2 in Fig. 5). This effect is specific to VgrG2b since induction of $vgrG2a_{HA\text{-}Strep}$ expression in HEK293 cells had no effect on uptake (Fig. 5, bar 14). Moreover, it is still dependent on microtubules since colchicine decreased the entry (4.3-fold) (compare bars 2 and 4 in Fig. 5).

To test if the intracellular pool of VgrG2b is sufficient to promote entry of bacteria impaired in internalization, we performed infections with invasion-deficient *P. aeruginosa* mutants lacking H2-T6SS or VgrG2b. Indeed both of these mutant strains were efficiently taken up by the HEK293 cells (bars 6 and 10 in Fig. 5), the intracellular production of VgrG2b increasing their uptake almost 2.8 times while maintaining dependency on microtubules (bars 8 and 12 in Fig. 5).

Altogether these data show that the intracellularly produced VgrG2b, presumably through interaction with tubulins and γTuRCs, can promote a microtubule-dependent entry of extracellular bacteria and even of bacteria that are normally impaired in invasion. This is another strong piece of evidence that VgrG2b delivery and *P. aeruginosa* uptake are two distinct steps.

DISCUSSION

A number of extracellular pathogens, including *C. jejuni*, *C. freundii*, *H. influenzae*, and *B. cepacia* complex, are able to invade nonphagocytic cells by modulating the dynamics of host microtubules. The basic mechanism of this important virulence-enhancing strategy is poorly understood. Here we demonstrated that upon infection by *P. aeruginosa*, the bacterial effector VgrG2b is delivered by the H2-T6SS machinery into epithelial cells, where it interacts with the α- and β-tubulins and γTuRC. This interaction facilitates the uptake of the bacterium, a process dependent on microtubule dynamics since colchicine and nocodazole, two microtubule-destabilizing agents, impaired the internalization. Importantly *P. aeruginosa* internalization still involves the actin cytoskeleton. We thus provide a key insight into the self-promoted invasion process by showing that a bacterial effector is delivered into the host cells from extracellular bacteria to target microtubule subunits and in particular γTuRC, a multiprotein complex that promotes nucleation of microtubules.

Two orphan VgrGs, VgrG2a and VgrG2b, were experimentally validated as belonging to the H2-T6SS machine. The main difference between the two proteins is the extreme C-terminus extension of VgrG2b, which is absent in VgrG2a (Fig. 1B). The strains lacking either of one of the VgrGs or the H2-T6SS displayed the same internalization impairment by HeLa or A549 cells (Fig. 1C; see Fig. S1B in the supplemental material), which confirms a connection between the effectors and this particular T6SS machine despite the absence of a physical linkage between their respective genes (Fig. 1A). However, it seems that only VgrG2b is delivered into host cells upon infection of epithelial cells (Fig. 2) and is able to interact with the α- and β-tubulins and γTuRCs (Fig. 3C and D; see Fig. S3 in the supplemental material). The focal organization of VgrG2b into host cells (Fig. 2A and 3B) and the fact that VgrG2b binds γ-tubulins under microtubule-depolymerizing conditions (Fig. 3D) indicate that the interaction of VgrG2b with the γTuRC is α- and β-tubulin independent and probably direct. However, the interactants of VgrG2b within the γTuRC still remain to be identified. The evolved VgrG2b therefore fulfills a dual function as a structural component of the secretion apparatus and a true effector binding γTuRC, whereas VgrG2a may be considered only as a structural VgrG of the H2-T6SS puncturing device. Such bifunctional VgrGs are also recovered in *V. cholerae*, where

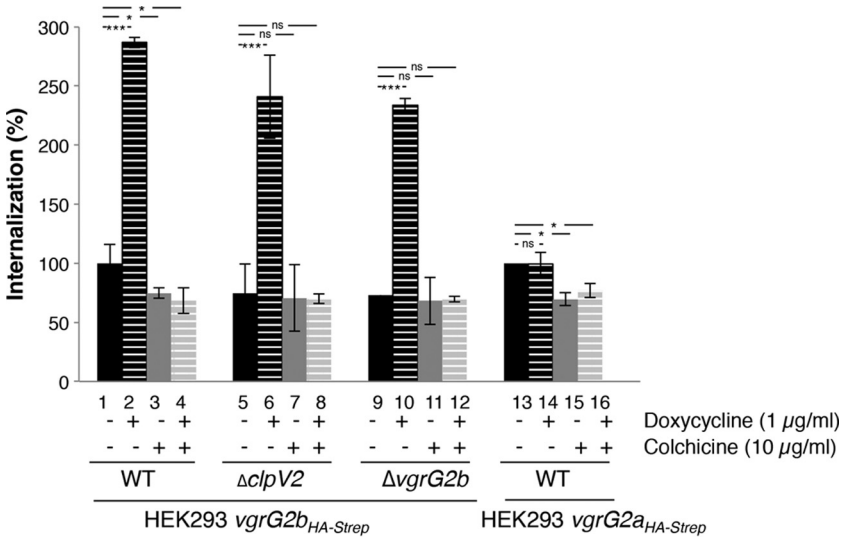


FIG 5 Bypassing the injection step. HEK293 cells overproducing (+ doxycycline) or not (− doxycycline) VgrG2b$_{HA-Strep}$ or VgrG2a$_{HA-Strep}$ were infected by different strains of *P. aeruginosa* in the presence (+) or not (−) of 10 μg/ml colchicine. Doxycycline (1 μg/ml) was added during 16 h for the induction of *vgrG2a*$_{HA-Strep}$ and *vgrG2b*$_{HA-Strep}$. The cells were then infected during 3 h at a MOI of 10. The percentage of invasion was normalized to PAO1, which represents an average of 2.8×10^5 CFU of internalized bacteria per well (2.5×10^5 HEK293 cells). Each experiment was performed a minimum of three times in triplicate. The error bars indicate standard deviations. ***, $P \leq 0.001$; *, $P \leq 0.05$. ns, not significant.

VgrG1 cross-links actin of the target cell through its C-terminal extension (32) and VgrG3 degrades the peptidoglycan of neighboring bacteria (28, 29, 34).

The work reported here addresses the role of bacterial and host cellular factors during *P. aeruginosa* internalization and describes a role for VgrG2b in this process through its α- and β-tubulin-independent interaction with γTuRC, the regulator of microtubule nucleation (42). The centrosome is the main site for microtubule nucleation, but most differentiated cells, including epithelial cells, possess nonradial microtubules arranged in noncentrosomal arrays (47). These nonradial microtubules are aligned along the apical-basal axis of the cell; the plus ends are located basally, whereas the minus ends are apical, thus contributing to the structural and functional polarity of epithelia (48). One could consider an enticing hypothesis whereby *P. aeruginosa* affected the polarity of the cell by recruiting γTuRC to sites of infection at the plasma membrane, in order to create new sites of microtubule nucleation. These new sites would be enriched in microtubule-minus ends, which might interfere with the directional transport of microtubule-dependent cargoes in the cell and thus affect the polarity of the cells (19). One type of cargo could be PI3K, whose recruitment to the apical surface may generate local production of PIP_3, creating a basolateral environment at the apical surface and formation of protrusions enriched for PIP_3 and actin. Indeed membrane-associated γ-tubulins were found to interact with kinases, among which is PI3K (49). In agreement with this, VgrG2b does not colocalize with the centrosome upon infection of HeLa cells or during intracellular production in HEK293 cells (see Fig. S2 and S5 in the supplemental material), suggesting that VgrG2b may interact with noncentrosomal γTuRCs. An example for noncentrosomal microtubule rearrangement during phagocytosis can be found in nonelicited macrophages, where microtubules are seen extending into the phagosomal cup, in addition to actin cytoskeleton rearrangements (50). Accordingly, colchicine treatment reduces pseudopod formation but also causes a decrease in activated PI3K and Akt (50). Altogether, the findings by Khandani et al. suggest that microtubules regulate the recruitment and localized activity of PI3K and Akt during pseudopod formation. Upon host kinase activation by contact with *P. aeruginosa*, VgrG2b might then promote microtubule nucleation at the membrane through interaction with γTuRCs that would lead to pseudopod formation for engulfment of the bacterium. One would expect to find noncentrosomal microtubule remodeling during *P. aeruginosa* internalization in epithelial cells, which we have been unable to observe yet. In concordance with this hypothesis, *Salmonella enterica* serovar Typhimurium invades fibroblasts by a route differing from the entry into epithelial cells (51). Uptake of *S. enterica* by fibroblasts requires microtubules and PI3K activation in a type III secretion system (T3SS)-independent way, whereas microtubules and PI3K are dispensable during *Salmonella* T3SS-based invasion of epithelial cells and macrophages.

This raises the question regarding the nature of the C-terminal effector domain of VgrG2b and the phylogenetic distribution of VgrG2b-like proteins. VgrG2b is conserved in all *P. aeruginosa* strains present in the *Pseudomonas* genome database. Moreover, a sequence similarity search indicated a strong association of VgrG2b with the *Pseudomonas* genus. To identify VgrG2b homologues that possess the C-terminal extension, we developed an *in silico* 3-step strategy (see Text S1 and Fig. S6 in the supplemental material). The phylogenetic tree clearly separated two groups, the VgrG2b of *Pseudomonas* and those of *Burkholderia* species, even if other few species are represented, like *Acinetobacter* (see Fig. S6B and C). Interestingly, all of these proteins contained the DUF2345 domain (PF10106), whereas this criterion has not been included in the search (see Text S1 and Fig. S6A and B), suggesting that this domain is a hallmark of VgrG2b homologues. Unexpectedly, the putative zinc protease domain predicted in VgrG2b (32) was not present in *B. cepacia* complex (BCC) homologues and seems thus restricted to *Pseudomonas* (see Fig. S6A and B). Interestingly *P. aeruginosa* and BCC are both recognized as respiratory patho-

gens in cystic fibrosis patients. Bacteria residing in the airway lumens proliferate in spite of host attempts to clear them through macrophage and epithelial cell uptake, neutrophil killing, and mucociliary clearance. Some BCC members (*B. cenocepacia* and *B. dolosa*) were shown to invade in a microtubule-dependent way the epithelial cells (8). Both pathogens may thus invade respiratory cells via a VgrG2b-mediated entry pathway, thus avoiding rapid elimination by macrophage phagocytosis or mucociliary clearance. Whether this leads to progression of infection is yet to be determined.

In the future, we will further address how *P. aeruginosa* subverts the microtubule network to invade nonphagocytic cells. The main questions that remain to be answered are what are the effects of the bacteria on cell polarity and recruitment of the microtubule motors and particularly whether this uptake leads to microtubule remodeling.

MATERIALS AND METHODS

Bacterial strains, plasmids, and antiserum production and growth conditions. All *P. aeruginosa* and *E. coli* strains used in this study are described in Table S1 in the supplemental material. See the experimental procedures in Text S1 in the supplemental material for the specific growth conditions, cloning procedures, and antisera used in Western blotting.

HeLa cell invasion assays. To enumerate bacteria internalized by HeLa cells through the H2-T6SS machinery, gentamicin protection assays were performed as described in reference 10 and modified in reference 20 with *P. aeruginosa* strains grown at the transition between the exponential and stationary phases. HeLa cells (ATCC, Manassas, VA) were cultured to 70% confluence on the day of the experiment in 24-well plates in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). The cells were washed twice with sterile phosphate-buffered saline (PBS), infected with a *P. aeruginosa* strain, and grown in the late exponential phase at a multiplicity of infection (MOI) of 10 in MEM with FBS at a final volume of 1 ml. Synchronous infection conditions and an enhanced bacterium-host cell interaction were achieved by a 5-min centrifugation step at 1,000 × *g*. After 3 h of infection at 37°C in 5% CO_2, the cells were washed twice with sterile PBS and incubated with MEM supplemented with 300 $\mu g \cdot ml^{-1}$ gentamicin (Sigma) for 1 h to kill extracellular bacteria. Afterward, the cells were washed three times with room temperature PBS and lysed at room temperature with 1% Triton X-100 (Sigma). Lysed cells were collected in Eppendorf tubes, and serial dilutions in sterile LB were plated onto *Pseudomonas* isolation agar plates. Colony-forming units were enumerated and reported as internalized bacteria.

To study the requirement of the microtubules or actin during bacterial internalization, colchicine (Sigma), nocodazole (Sigma), and cytochalasin D (Sigma) were added at various concentrations 30 min (colchicine) or 1 h (nocodazole and cytochalasin D) before infection with *P. aeruginosa*.

Immunofluorescence staining and image analysis. HeLa cells were plated onto the coverslip in DMEM complemented with 10% FBS medium at 150,000 cells/cm^2 and infected with *P. aeruginosa*, grown at the transition between the exponential and stationary phases, at an MOI of 10 for 3 h. After being rinsed with PBS three times, coverslips were washed and fixed with either 4% paraformaldehyde (PFA)–PBS for 10 min at room temperature or with methanol 100% at −20°C for 15 min. After three washes with PBS, the cells were permeabilized with PBS–0.1% Saponin for 30 min. The coverslips were blocked with 10% bovine serum albumin (BSA)-PBS for 1 h followed by incubations with primary (anti-V5 and anti-VgrG2b, 1/500) and secondary antibodies for 1 h each in 10% BSA–PBS at room temperature. Secondary antibodies were stained with the fluorescence-coupled Cy3- or Cy5-conjugated goat anti-rabbit (A11008) and Cy3- or Cy5-conjugated goat anti-mouse (A11005) antibodies (both from Invitrogen). After another three washes with PBS, coverslips were mounted on glass slides using ProLong Gold (Invitrogen) and left in the dark overnight before the microscopy study. Samples were examined with a confocal microscope (Olympus IX81) using a ×60 oil objective (UPLSAPO; numerical aperture, 1.35). Images were acquired using FLUOVIEW Viewer software.

Affinity purification. Full details of the construction and cell culture of isogenic stable cell lines exhibiting tetracycline (Tet)-inducible expression of recombinant *vgrG2a*, *vgrG2b*, and *gfp* genes can be found in Text S1 in the supplemental Material.

$VgrG2a_{HA\text{-}Strep}$, $VgrG2b_{HA\text{-}Strep}$, and $GFP_{HA\text{-}Strep}$ HEK293 Flp-In cell pellets were lysed by incubation in freshly prepared buffer (50 mM HEPES [pH 8.0], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 1.5 mM Na_3VO_4, 1.0 mM phenylmethylsulfonyl fluoride [PMSF], and protease inhibitor cocktail) on ice for 20 min. Each lysate was centrifuged at 14,000 × *g* for 30 min, and the supernatant containing the protein extract was collected. The protein concentration was determined by the Bradford assay using BSA as a standard. Two-step affinity purifications were performed as previously described (52) and detailed in the supplemental material.

Reversed-phase LC-MS. The protein eluates from the two-step affinity purifications were reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin. A complete protocol for LC-MS separation and data analysis is presented in the supplemental material.

Coimmunoprecipitation. HEK293 Flp-In T-REx cells carrying the genes for $vgrG2a_{HA\text{-}Strep}$ or $vgrG2b_{HA\text{-}Strep}$ were cultured in 10-cm petri dishes in Dulbecco's modified Eagle's medium (DMEM) according to the manufacturer's instructions (Invitrogen). The expression of recombinant *vgrG2a* or *vgrG2b* was induced by adding doxycycline to the medium to 1 μg/ml for 16 h. Subsequently, cells were scraped off the culture dish and centrifuged, and after two rinses with PBS, cells were lysed with 75 μl/dish of 50 mM HEPES, 100 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 1% NP-40, 1 mM β-mercaptoethanol, and a cocktail of protease inhibitors (Roche). Six microliters of ascites fluid containing monoclonal antibody against the HA tag was added (clone HA-7; Sigma-Aldrich), and following incubation for 30 min on ice, immunoprecipitation was performed by adding 50 μl of protein G coupled to magnetic beads (Dynabeads, Invitrogen). Following several rinses, bound protein was eluted from the beads with 40 μl of Laemmli gel electrophoresis buffer containing 2% SDS. Eluted proteins, as well as cell lysates before and after immunoprecipitation, were analyzed by Western blotting. Western blots were probed with mouse-anti-HA (clone HA-7; Sigma-Aldrich), and rabbit antibodies against γ-tubulin or GCP4, respectively (53).

Statistical analyses. Unpaired Student's *t* tests and the χ^2 test were performed using Excel software (Microsoft). On the figures, asterisks indicate statistical significance.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00712-15/-/DCSupplemental.

Text S1, DOCX file, 0.03 MB.
Figure S1, PDF file, 0.2 MB.
Figure S2, PDF file, 0.8 MB.
Figure S3, PDF file, 0.2 MB.
Figure S4, PDF file, 0.2 MB.
Figure S5, PDF file, 0.1 MB.
Figure S6, PDF file, 0.7 MB.
Table S1, DOCX file, 0.02 MB.

ACKNOWLEDGMENTS

We thank V. Baylot and members of R.V.'s team for constant support, B. Ize and S. Salcedo for careful reading of the manuscript, P. Hubert for training and advice on confocal microscopy, E. Cascales for providing HA-tagged antibodies, and J. van Helden for advice on statistical analysis.

T.G.S. was financed with a Ph.D. fellowship from the French Research Ministry and with a "Teaching and Research" fellowship from AMU, and A.H. was financed with a postdoctoral fellowship from EuroPathoGenomics and Wellcome Trust (WT091939). This work was supported by

"Pathomics" ERA-net PATHO grant ANR-08-PATH-004-01. A.F.'s group was financed by MRC grant MR/K001930/1.

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